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Editorial Comment

Significance of Linkage Disequilibrium Between the Fragile X Locus and Its Flanking Markers

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INTRODUCTION

The identification of several microsatellite markers flanking the FRAXA locus [Richards et al., 1991a,b; Riggins et al., 1992] was instrumental in the positional cloning of the FMR1 gene. These markers can still be valuable in family studies, e.g., as additional evidence in prenatal diagnosis. Additionally, they were employed to verify the presence of any significant gametic disequilibrium between the fragile X mutation and some haplotypes, although the high mutation rate predicted from early segregation studies [Sherman et al., 1985] implied that new mutants would arise on almost every chromosomal background. Thus, the discovery of linkage disequilibrium encompassing the fragile X locus has been surprising. Here, we review the available evidence of such gametic association and underline its implications for the mutational mechanism.

FRAGILE X-ASSOCIATED HAPLOTYPES

Tables I and II summarize allelic frequencies at two of the most investigated loci (DXS548 and FRAXAC1) in various fragile X and control populations, mainly of European descent [Richards et al., 1992; Hirst et al., 1993; Jacobs et al., 1993; Buyle et al., 1993; Oudet et al., 1993a; Haataja et al., 1994; Malmgren et al., 1994; Macpherson et al., 1994; Zhong et al., 1994a; Chiurazzi et al., 1996a] or of Asiatic origin [Richards et al., 1994a]. In all cases it is apparent that although fragile X patients can display several haplotypes, only a few account for almost 70–80% of the total, with a distribution significantly different from that of controls. Moreover, isolated populations such as the Finns [Oudet et al., 1993b; Haataja et al., 1994; Zhong et al., 1996] and Swedes [Malmgren et al., 1994] show an even more pronounced effect, with one single dominant haplotype shared by most patients. We also note that the main fragile X haplotypes are common to the different European nations and seem distributed along geographical gradients. For example, the FRAXAC1-A/DXS548-2 haplotype, which is prevalent in Italy, and the FRAXAC1-D/DXS548-6 haplotype, prevalent in the UK, are almost equally frequent in France. Preliminary studies have also investigated the haplotypes of normal Chinese [Zhong et al., 1994b] and black Africans [Chiurazzi et al., 199b]. In accordance with genome-wide microsatellite surveys [Deka et al., 1995], Africans show the highest heterozygosity and mean allele number, possibly suggesting that they have had the longest evolutionary history, while Asiatic populations have the least genetic diversity and lack the European founder haplotype FRAXAC1-A/DXS548-2.

CGG REPEAT STRUCTURE

Recent surveys [Kunst and Warren, 1994; Hirst et al., 1994; Eichler et al., 1994, 1996; Snow et al., 1994; Zhong et al., 1995; Rousseau et al., 1995] have focused on the normal control population and on the correlation between haplotypes and CGG length and internal structure. The presence and position of intercalating AGGs seem to be a major determinant of triplet-repeat stability [Hirst, 1995]. In controls, it was shown that one principal fragile X haplotype (FRAXAC1-A/DXS548-2, or 2-1-3 of Eichler et al. [1996]) is associated with longer-than-average arrays which have a variable 3′ uninterrupted tract, while retaining the two most 5′ AGG interruptions (e.g., 9A9Ax, where x > 20), and seem to constitute a pool of more unstable triplets. The eight-generation family reported by Smits et al. [1993], with the DXS548-2 allele in 5 probands, may be an example of this instability. On the other hand, the other most prevalent fragile X haplotypes (FRAXAC1-D/DXS548-6, or 6-4-4 and 6-4-5 of Eichler et al. [1996]) are not associated with repeats of longer total size in the control population, but either lack the second AGG interruption (e.g., 9A22 or 9A26) or have a slightly larger middle CGG tract (e.g., 9A11A9 or 9A12A9). In this case, a more rapid transition to the premutation (S-to-Z, according to Morton and Macpherson [1992]) appears to take place, possibly after loss of the second stabilizing AGG.

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TABLE I. Allele Distribution at Locus DXS548 in Different Fragile X and Control Populations*

Reference and country	Zhong et al. [1994a], USA		Macpherson et al. [1994], UK		Buyle et al. [1993], Belgium, Holland		Oudet et al. [1993a], France		Chiurazzi et al. [1996a], Italy		Malmgren et al. [1994], Sweden		Haataja et al. [1994], Finland		Zhong et al. [1994b], China
Allele	Fraxa	Contr.	Fraxa	Contr.	Fraxa	Contr.	Fraxa	Contr.	Fraxa	Contr.	Fraxa	Contr.	Fraxa	Contr.	Contr.
1	1.6	1.6	2.3	4.3		3	0.9	1.2	5.6	1.9				2	
2	16.8	7.9	18.2	6.4	36.8	10.4	27.5	9.3	28.8	4.7	3.6	7.1	8	10	
3	8.8	3.7				0.8	0.9	0.6	9.6					3	
4	4.8				1.5	1.5	0.9	0.6	1.6						0.4
5	0.8	1.1								1.4					1.3
6	20.8	9.4	36.3	14.9	20.6	10.4	30.2	14.2	19.2	13.9	46.4	14.3	90	16	13.2
6.5									0.8						
7	39.2	73.1	34.1	73.3	39.6	73.1	39.6	72.3	28.8	75.3	50	78.6	2	69	80.7
8	7.2	3.2	9.1	1.1	1.5			1.8	5.6	1.9					4.4
9						0.8				0.9					
Chromosome number	125	190	44	188	68	134	106	162	125	215	28	28	60	283	227

* Frequencies are given in order to facilitate comparison, while absolute numbers of chromosomes tested are indicated in last line. Allele names are as in Macpherson et al. [1994].

TABLE II. Allele Distribution at Locus FRAXAC1 in Different Fragile X and Control Populations*

Reference and country	Zhong et al. [1994a], USA		Richards et al. [1992]		Hirst et al. [1993]		Macpherson et al. [1994], UK		Jacobs et al. [1993], UK		Chiurazzi et al. [1996a], Italy		Richards et al. [1994a], Japan		Zhong et al. [1994b], China
			Australia	UK, Ceph	UK, Belgium	UK, Ceph									
Allele	fraxa	contr.	fraxa	contr.	fraxa	contr.	fraxa	contr.	contr.	fraxa	contr.	fraxa	contr.		contr.
Z (0)									0.7						
A (1)	28.8	3.7	21	7	27.4	4.6	27.3	5.3	7	31.4	6				
B (2)	3.2	5.8	2	2	5.5		9.1		0.7	0.7	2.1				
C (3)	33.6	66.8	32	74	38.4	78.5	29.6	75.5	74	39.5	68.9	60		31	29.2
D (4)	34.4	22.1	45	17	28.9	16.2	34	19.2	17.3	27	23	40		69	70.8
E (5)		1.6				0.7			0.3	0.7					
F (6)										0.7					
Chr.n	125	190	47	202	73	130	44	188	304	137	235	40		142	216

* Frequencies are given in order to facilitate comparison, while absolute numbers of chromosomes tested are indicated in last line. Allele names are as in Richards et al. [1992], while numbers in parentheses are as in Macpherson et al. [1994].

GAMETIC ASSOCIATION AND MULTISTEP MUTATIONAL PATHWAYS

It is evident that a correct interpretation of linkage disequilibrium and CGG structure data depends on an understanding of the mutational mechanisms responsible for the instability of the FMR1 CGG repeat. Almost two thirds of fragile X full mutations are associated with few (4–5) haplotypes: this is surprising, given the estimate of the overall mutation rate of 0.8×10^{-4}. This rate of mutation from the normal stable to abnormal unstable state is based on the recently reevaluated prevalence of affected males (1/4,000 according to Turner et al. [1996]) and the assumption of equilibrium. Such an estimate appears too high for founder chromosomes to be detectable after several generations. As correctly pointed out by Chakravarti [1992] and Kolehmainen [1994], it is difficult to reconcile this high frequency of new mutations with the suggestion that most fragile X mutations arose on a small number of chromosomes. Actually, hundreds of "founder" alleles per million meioses generated at random would have produced a fragile X haplotype distribution almost identical to that of controls. A better explanation would be that of a preferential occurrence of mutations "on particular chromosomal backgrounds" [Kolehmainen, 1994].

The far-reaching hypothesis of a multistep mutational pathway (Fig. 1a), leading to the constitution of pools of "intermediate" alleles (e.g., N, S, Z, L, and possibly other alleles) with distinct identities and con-

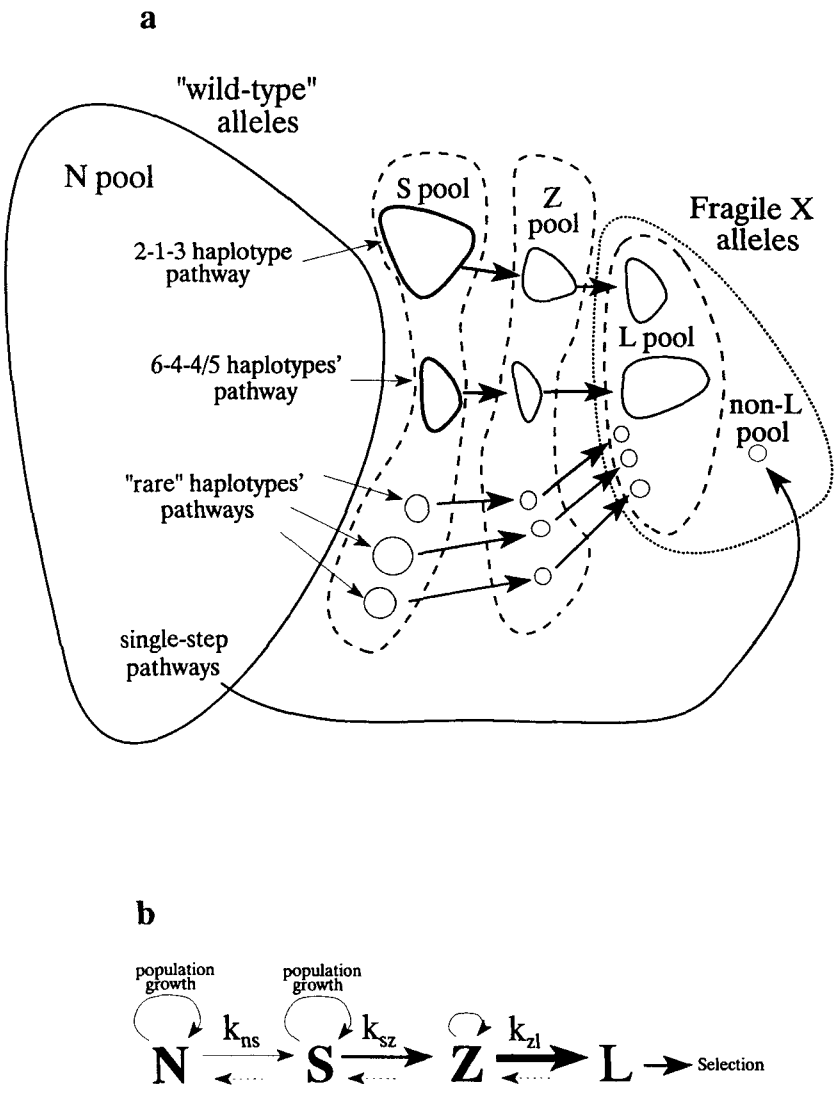

Fig. 1. **a:** Multiple mutational pathways leading from "wild-type" alleles (N pool) to fragile X alleles. Single-step events (traditional mutations, such as deletions and point mutations) lead to nonfull-mutation (non-L) fragile X, while multistep processes lead to more prevalent full mutations (L pool). S and Z intermediate pools are also indicated. **b:** One multistep mutational pathway represented as a chain of biochemical reactions, each one corresponding to a different mechanism with its peculiar transition rates. Note that reverse reactions have almost undetectable rates, and that the pathway is in a state of constant forward flow (i.e., the biochemical steady-state equivalent to mutation-selection "equilibrium").

nected by different mechanisms, each operating at its peculiar rate, was formalized by Morton and Macpherson [1992] and then incorporated in all other models [Kolehmainen, 1994; Morris et al., 1995a,b; Ashley and Sherman, 1995]. A useful analogy can be drawn with a biochemical pathway (Fig. 1b), and we note that the *absolute* number of alleles transferred from one to the next pool is the same "at equilibrium" (i.e., at steady flow), but the *relative* frequency of transition is different because the size of the originating pool is different. The analogy also readily explains that the S "pool" is larger than the Z "pool" because the S-to-Z transition rate is lower than the Z-to-L rate (in fact, Z alleles are readily converted into L alleles). Thus, the different sizes of the pools of the intermediate "products" (i.e., their particular prevalence in the population) make up for differences between individual transitional rates between one pool and the other. This allows the coexistence of low-frequency initial events (accounting for the gametic association) and the prevalence of fragile X. One way to reconcile the relatively high mutation rate and the observation of haplotype association is to consider several different mutational pathways (Fig. 1a). For example, one pathway could include the loss of an AGG, which is probably due to a point mutation. This point mutation may only occur at a rate of 10^{-5}–10^{-7}, and therefore it may be associated with a few founder chromosomes. Another pathway may begin with replication slippage, leading to an increase of perfect repeats (e.g., on 2-1-3 chromosomes, as suggested by Eichler et al. [1996]). Thus, although the overall mutation rate of 0.8×10^{-4} is too high to result in founder effects, some steps of each underlying pathway could occur at much lower rates and could be expected to be associated with specific backgrounds. Genetic drift of the neutral S alleles would play a large role in creating "reservoir" pools. Eventually, other mechanisms (e.g., Okazaki fragment slippage at both ends) could account for the transition to larger premutations or full mutations [Richards and Sutherland, 1994] at a much faster rate, estimated at between 1×10^{-2} and almost 0.8 per generation, at least when the Z allele is transmitted from the mother.

ORIGIN OF FRAGILE X CHROMOSOMES

As shown in Figure 1a, the fragile X prevalence of 1 in 4,000 males is probably an endpoint obtained from different "pathways." A small percentage of non-L (non-full mutation) fragile X alleles are due to traditional single-step mechanisms (e.g., deletions or point mutations), which are necessarily short-lived and not preferentially associated with any haplotype.

Roughly two thirds of the full-mutation (L) fragile X chromosomes are in linkage disequilibrium with specific haplotypes for the reasons discussed above. A single ancestral chromosome with haplotype 2-1-3, associated with a 9A9Ax triplet array, might account for 15–25% of fragile X chromosomes. The N-to-S transition event in this case may have been the growth by a few units of the 3′ CGG tract by a slipped-strand mispair mutation mechanism [Levinson and Gutman, 1987; Weber and Wong, 1993; Di Rienzo et al., 1994], additionally constrained by conservation of the first two AGGs. Alternatively, it may have been a 29-to-39 "jump" (from 9A9A9 to 9A9A9A9, as in Macpherson et al. [1995]) followed by the loss of an AGG (9A9A9A9 to 9A9A19).

In the case of haplotypes 6-4-4 and 6-4-5, the N-to-S event is probably a particular "symmetric" interspersion pattern (9AxA9, with $x > 10$), as suggested by Eichler et al. [1996]. This might predispose to the rapid loss of the second AGG (the S-to-Z transition of this pathway), and we note that no longer-than-average CGG alleles are associated with these haplotypes in the control population (which contains both N and S alleles, while Z and L alleles can be distinguished by their size). Actually, this may be because the S-to-Z transition rate on this pathway is higher than that on the 2-1-3 haplotype pathway, and does not allow the formation of a large detectable "pool" of S intermediates (Fig. 1a).

We note that some low-frequency haplotypes are likely to derive from these few "major" fragile X-associated haplotypes by recombination and/or mutation at the microsatellites themselves. In fact, during the several generations after the initial mutation, the more highly mutable marker microsatellites may transform a "major" haplotype into a set of apparently "new" ones, clustered next to it [Chiurazzi et al., 1996a]; this could also partially account for the high heterozygosity observed in fragile X samples. The analysis of the less informative but more stable single nucleotide polymorphisms, FMRa and FMRb [Kunst and Warren, 1994], might be appropriate in resolving this particular issue.

A local instability mechanism has been suggested by Zhong et al. [1994a] to explain linkage disequilibrium: here the initial amplification steps at the CGG repeat would predispose the flanking markers to instability, but this should happen in a "directional" way (possibly in the sense of a size increase), so that otherwise no preferential association would be detected. Anyhow, at this time, no such mechanism has been identified.

The remaining third of fragile X full mutations does not show any significant linkage disequilibrium: these mutations are mainly associated with less frequent haplotypes, which may be recent mutations that have not yet attained high frequencies, sometimes with unique "rare" haplotypes, not observed on any control chromosome [Macpherson et al., 1994]. It is possible that some external factor, perhaps a mutation at a DNA repair locus, gives rise to simultaneous instability in FMR1 and its flanking loci. If this happened in an individual homozygous for an instability mutation [Brown et al., 1996], an unpredicted "rare" haplotype would be stabilized in the heterozygous offspring. As yet, no "mutator" phenotype has ever been observed in vivo or in vitro in fragile X families (normal life span, no specific familial cancers), although exceptional pedigrees might, by coincidence, happen to show a genome-wide instability. Such a family was described by Zhong et al. [1993], where three FRAXAC2 mutations were observed, one of which was significantly not on the fragile X chromosome [Richards et al., 1994b; Mornet et al., 1994; Zhong et al., 1994c]. Surveys of isolated normal populations [Eichler and Nelson, 1996; Chiurazzi et al.,

1996b; Kunst et al., 1996] indicate that the FMR1 triplet array attained its average size before the spread of *Homo sapiens,* thus making gross differences in fragile X prevalence among various ethnic groups unlikely. Nevertheless, the particular AGG interspersion pattern in small and isolated populations, such as some Native Americans [Kunst et al., 1996], might indeed stabilize the CGG triplet over hundreds of generations. Other population surveys investigating the size and purity of FMR1 triplet arrays will be needed to refine our understanding of the first amplification steps, also bearing in mind the possibility of size reductions [Vits et al., 1994; Chiurazzi et al., 1994], and also to help us understand the relative roles of hairpin formation and replication slippage on the leading and/or lagging strand. Extended family analysis will be necessary to clarify the behavior of "gray zone" alleles.

NOTE ON COMPLEX MICROSATELLITES AND ON NOMENCLATURE

Detection of linkage disequilibrium can be less efficient when inadvertently using a complex marker microsatellite, i.e., a marker including more simple repeats in the same amplification product; in this case alleles cannot be distinguished unequivocally by their size, and exact typing would require laborious sequencing, as in the case of FRAXAC2, whose complex structure, (GT)xC(TA)y(T)z, was elucidated by Zhong et al. [1993]. A much less polymorphic secondary sequence may be also present in the DXS548 PCR product [Chiurazzi et al., 1996a,b], but in this case it was possible to realize a new 5′ primer that dissects the primary CA repeats and allows a straightforward PCR size-typing [Chiurazzi et al., 1996a]. The involvement of many independent research groups has created discrepancies in nomenclature for microsatellite alleles. An attempt to draw together the various systems currently in use was reported by Macpherson et al. [1994] in their Table 1. Systems based on absolute size of PCR products should be avoided, as they suffer from the variability of DNA migration at electrophoresis, and become confusing when new primers are used to amplify the same sequence. On the other hand, systems based on arbitrary letter or number codes, which can be standardized by circulating reference DNAs, are easier to employ, although they do not refer to any actual sequence information. In theory, systems based on the number of repeats would be most satisfying, and possibly a two-digit format with a reference point 00 for zero repeats could allocate both even and odd microsatellite alleles. Such a format would be easy to use, given the availability of a standard set of reference DNAs which could be circulated between laboratories. In the absence of any consensus, the increasing body of data may eventually become impossible to collate and compare.

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